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Production of a Hemolytic Factor by *Candida albicans*

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***Candida albicans* exhibits hemolytic activity when grown on glucose-enriched blood agar. This activity is present on intact organisms, and it is secreted into the culture medium. Hemoglobin released from lysed erythrocytes can restore the transferrin-inhibited growth of *C. albicans*. We conclude that *C. albicans* expresses a hemolytic factor which allows it to acquire iron from host erythrocytes.**

Candida albicans, a dimorphic fungus which normally inhabits the human gastrointestinal system, is a major cause of opportunistic fungal infection in the immunocompromised host (14). The ability of pathogenic organisms to acquire iron in the mammalian host has been shown to be of critical importance in establishing infection (1, 15, 16, 19). In humans, most of the iron is located intracellularly as ferritin or as heme-containing compounds. The small amount of extracellular iron is attached to the iron-binding and transport proteins transferrin and lactoferrin. Therefore, since there is essentially no free iron in the body, pathogens must acquire it from one or more iron-containing compounds (15).

It has previously been shown by several groups that the growth of *C. albicans* can be inhibited in human serum (3, 6, 13, 17). It has been further demonstrated that this growth inhibition is due to the presence of the serum protein transferrin (3, 5, 7, 13). The expression of complement-binding mannoproteins on germ tubes, pseudohyphae, and hyphae of *C. albicans* but not on yeast cells has been reported. These cell surface proteins are functional homologs of the mammalian complement receptors type 2 and type 3 (2, 4, 8, 9, 12). *C. albicans* can utilize these proteins to rosette complement-opsonized sheep erythrocytes (SRBCs) (4, 8, 13). We have previously shown that the binding of complement receptor type 3-like molecules to iC3b-coated SRBCs results in enhanced growth of hyphae under conditions of iron limitation (13). We have undertaken a study to determine if *C. albicans* produces a hemolytic factor by which it can acquire erythrocyte-derived iron for growth.

To determine which iron-containing compounds could restore the transferrin-inhibited growth of *C. albicans*, ferritin, hemin, and hemoglobin (Sigma Chemical Co., St. Louis, Mo.) were added to cultures at equimolar concentrations (Fig. 1). *C. albicans* (ATCC 32354) was passaged weekly on Sabouraud dextrose agar plates. Yeast cells that were grown overnight on this agar at 25°C were washed in sterile phosphate-buffered saline (PBS) and resuspended in RPMI 1640 (GIBCO Laboratories, Grand Island, N.Y.) supplemented with glutamine, penicillin, and streptomycin. Human transferrin (Sigma) at a concentration of 25 μg/ml, 30% iron saturated (15), was added to all cultures except the controls. Ferritin, hemin, and hemoglobin were added to cultures at a final concentration of 0.66 μM. Yeast cells were plated at 250 cells per well in 96-well polystyrene microtiter plates (Falcon) and incubated at 37°C in 5% CO_2 for 18 h. *C. albicans* cultured under these conditions converts from the yeast to the hyphal form. The extent of *Candida* growth was determined by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) viability assay (11) as previously described (12). The increase in the reduction of MTT to MTT formazan (1-[4,5-dimethylthiazol-2-yl]-3,5-diphenylformazan) reflects the increase in the growth of the organism.

The growth of *C. albicans* is dramatically inhibited in the presence of transferrin (Fig. 1). The organism can utilize iron derived from either ferritin, hemin, or hemoglobin. However, hemoglobin-derived iron restores *Candida* growth more efficiently than do the other two compounds tested (Fig. 1). The result for growth of *C. albicans* in RPMI 1640, a medium which contains sufficient iron to allow maximal *Candida* growth, is included for comparison.

Since *C. albicans* can utilize iron contained in hemoglobin and can rosette complement-opsonized SRBCs, a hemolysis assay was developed to determine if *C. albicans* produces a hemolytic factor to lyse erythrocytes. Although the presence of hemolytic activity by *C. albicans* has been previously reported (10, 18), it has been the subject of considerable controversy.

Hyphal cultures were prepared by suspending 2×10^8 yeast cells per ml in RPMI 1640 medium without phenol red and incubating the suspension at 37°C for 18 h at 150 rpm on a rotary shaker. Hyphae or yeast cells were washed three times in sterile PBS, aliquoted in 500-μl Eppendorf tubes, centrifuged at top speed in a microcentrifuge for approximately 20 s, and resuspended in 100 μl of sterile PBS. A hemolysis assay mixture was prepared by diluting complement-opsonized SRBCs (13) or unopsonized SRBCs to 10^7 cells per ml in PBS. A 200-μl aliquot of the diluted SRBCs was added to the Eppendorf tubes with the *C. albicans*. The tubes were centrifuged for 20 s to pellet the cells and then incubated at 37°C in 5% CO_2 for 2 h. The pellet was resuspended by gentle pipetting and then centrifuged again. The optical density at 405 nm of 200 μl of the supernatant was determined with a microELISA reader. The experiment was done in triplicate, and the average optical density was used to calculate the percent hemolysis (Fig. 2). The results indicated that *C. albicans* can lyse opsonized SRBCs more readily than unopsonized SRBCs and that the hyphal form shows greater hemolytic activity than does the yeast form. Cultures grown in the presence of transferrin, i.e., under conditions of iron deprivation, showed no increase in hemolytic activity. Similar levels of hemolytic activity were obtained with *C. albicans* Ru-7 (a gift from R. Rüchel, Hygiene Institut der Universität, Göttingen, Germany), a strain which produces acid proteinase, and IR24

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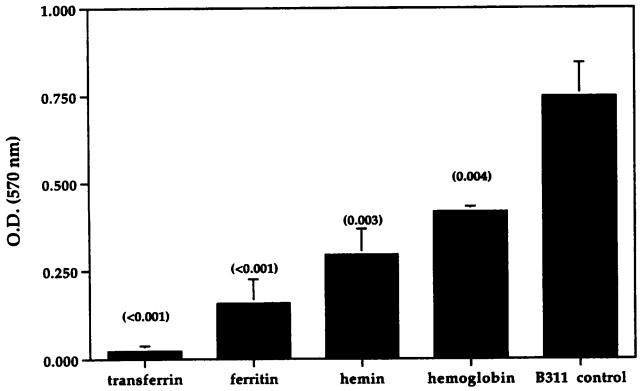

FIG. 1. Restoration of transferrin-inhibited growth by iron compounds. Transferrin-inhibited growth of *C. albicans* could be restored with a variety of iron compounds. *C. albicans* was grown in RPMI 1640 medium supplemented with 25 μg of transferrin per ml. Ferritin, hemin, and hemoglobin were added at a final concentration of 0.66 μM. No transferrin was added to the medium for the B311 control group. Results shown are representative of three experiments and are expressed as optical density (O.D.) at 570 nm (mean ± standard deviation) of MTT formazan from triplicate cultures. *P* values (in parentheses) are expressed relative to controls.

(R. Rüchel), an acid proteinase-deficient mutant (data not shown).

We have also observed that *C. albicans* ATCC 32354, as well as other strains and species of *Candida*, shows hemolytic activity when grown on enriched blood agar. Blood agar plates were prepared by adding 3 ml of washed SRBCs suspended in sterile PBS to 100 ml of agar which had been enriched with 3% glucose (wt/vol). Either Trypticase soy agar or Sabouraud dextrose agar was used. A distinct ring of lysis was observed around colonies that were incubated at 37°C in 5% CO_2 for 24 to 48 h (Fig. 3). No lysis occurred when there was no glucose in the medium or when cultures were grown at temperatures other than 37°C (data not shown).

To determine if glucose is required for the lytic effect, several different sugars were added to Trypticase soy agar and the hemolytic activity was observed. *C. albicans* ATCC 32354,

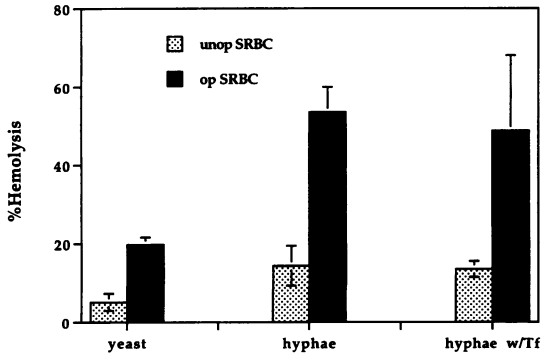

FIG. 2. Hemolytic activity of *C. albicans*. Yeast cells, hyphae, and transferrin-inhibited (w/Tf) hyphae were tested for their ability to lyse complement-opsonized (op) and unopsonized (unop) SRBCs. The percent hemolysis was determined by comparing the optical density at 405 nm of the test group with that of 100% lysis with water. Results shown are representative of three experiments done in triplicate and are expressed as means ± standard deviations.

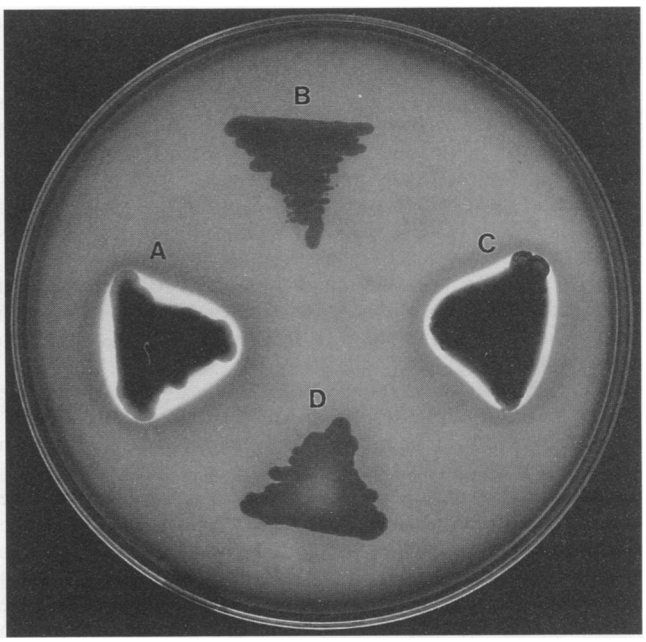

FIG. 3. Growth of *C. albicans* on enriched blood agar. *C. albicans* ATCC 32354 (A) and strain V6 (C) show a ring of lysis around colonies streaked on glucose-enriched blood agar, whereas *Cryptococcus neoformans* (B) and *Candida parapsilosis* (D) do not. Cultures were incubated for 24 to 48 h at 37°C in 5% CO_2.

V6 (a variant of ATCC 32354 which does not form hyphae under these conditions), and two clinical isolates grown on blood agar plates containing glucose or maltose demonstrated hemolytic activity. There was no hemolysis on plates contain-

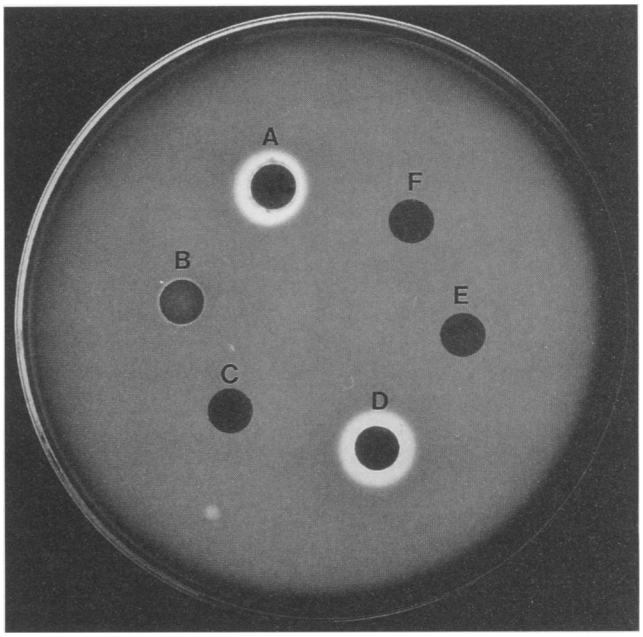

FIG. 4. Secretion of a hemolytic factor by *C. albicans*. Sterile filter paper disks were saturated with the supernatants of overnight cultures of *C. albicans* ATCC 32354 (A); *C. albicans* V6 (B); *Cryptococcus neoformans* (C); a positive control, group A streptococcus (D); and group B streptococcus (E). RPMI 1640 medium alone (F) served as a negative control. Plates were incubated for 24 h at 37°C in 5% CO_2.

ing sucrose, lactose, or galactose in the medium. Medium containing 2-deoxy-2-glucose inhibited the growth of the organism.

The factor that is responsible for this hemolytic activity is secreted into the growth medium. Sterile filter paper disks were saturated with supernatants of overnight cultures inoculated with 2×10^8 cells per ml. A clear ring of hemolysis surrounded disks which were soaked in culture supernatants from *C. albicans* ATCC 32354, and the radius of this ring was comparable to that of the ring surrounding the positive control, group A streptococcus. A smaller hemolysis zone surrounded the disk containing the culture supernatant from the V6 variant and may correlate with the inability of this strain to form hyphae (Fig. 4).

The findings presented here indicate that *C. albicans* has the ability to utilize iron derived from hemoglobin and that *C. albicans* may acquire this iron by producing a factor which can release hemoglobin by lysing erythrocytes. Production of this hemolytic factor may be regulated by the presence of glucose in the growth medium. In the present report, we define the conditions under which *C. albicans* can produce a hemolysin and we demonstrate that the activity is present on the intact organism and can be secreted into the culture medium. We are currently investigating the nature of this hemolytic factor in order to determine its importance as a putative virulence factor.

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